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Evaluation of a Strategy for *Toxoplasma gondii* Oocyst Detection in Water

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Several recent outbreaks of toxoplasmosis were related to drinking water. We propose a strategy for *Toxoplasma* oocyst detection as part of an approach to detecting multiple waterborne parasites, including *Giardia* and *Cryptosporidium* spp., by the U.S. Environmental Protection Agency method with the same sample. Water samples are filtered to recover *Toxoplasma* oocysts and purified on a sucrose density gradient. Detection is based on PCR and mouse inoculation (bioassay) to determine the presence and infectivity of recovered oocysts. In an experimental seeding assay with 100 liters of deionized water, a parasite density of 1 oocyst/liter was successfully detected by PCR in 60% of cases and a density of 10 oocysts/liter was detected in 100% of cases. The sensitivity of the PCR assay varied from less than 10 to more than 1000 oocysts/liter, depending on the sample source. PCR was always more sensitive than mouse inoculation. This detection strategy was then applied to 139 environmental water samples collected over a 20-month period. Fifty-three samples contained PCR inhibitors, which were overcome in 39 cases by bovine serum albumin addition. Among 125 interpretable samples, we detected *Toxoplasma* DNA in 10 cases (8%). None of the samples were positive by mouse inoculation. This strategy efficiently detects *Toxoplasma* oocysts in water and may be suitable as a public health sentinel method.

Toxoplasma gondii is a protozoan parasite capable of infecting a variety of birds and mammals, including humans (40). Toxoplasmosis is a significant problem in congenitally infected infants and immunosuppressed patients. *T. gondii* is transmitted in three main ways: (i) from mother to fetus, (ii) by consumption of undercooked meat containing tissue cysts, and (iii) by ingestion of food or water contaminated by sporulated oocysts. Members of the Felidae are the only known hosts that can release oocysts into the environment in their feces (11). Excreted oocysts are unsporulated and are not directly infectious. They become infective after 1 to 21 days in the external environment, depending on aeration and temperature (9). Although cats excrete oocysts over only short periods (1 to 3 weeks), they release massive numbers of oocysts (10^7/10^8), which are highly resistant and can survive for months in the environment (13).

Three waterborne toxoplasmosis outbreaks have been documented (2–4). The first occurred in Panama in 1979 (3). An epidemiological investigation identified the source as creek water contaminated by oocysts excreted by jungle cats. A large waterborne outbreak occurred in British Columbia (Canada) in 1995, with 110 cases of human acute *Toxoplasma* infection (4). The source was municipal drinking water, probably contaminated by cougar and/or domestic cat feces (1). The largest outbreak, with 290 human cases, was recently reported in Brazil and involved an unfiltered water reservoir (22). Bahia-Oliveira et al. found a high *T. gondii* prevalence in a Brazilian community, related to drinking unfiltered water (2). Likewise, Hall et al. identified drinking water as the probable source of infection in a community of strict vegetarians (18).

Waterborne transmission of the intestinal parasites *Giardia duodenalis* and *Cryptosporidium parvum* is also well documented (16, 36, 37). Over 160 waterborne outbreaks of giardiasis and cryptosporidiosis have been reported, and well-documented cases have been described in the United States and United Kingdom (26, 27, 29). *T. gondii* oocysts are resistant to the usual processes used to disinfect drinking water (42). Increase awareness of the risk of waterborne toxoplasmosis outbreaks has led to a search for methods that can efficiently detect oocysts in environmental water.

Here, we evaluated a strategy used since 2001 in our laboratory to detect *T. gondii* oocysts in water, along with *Giardia* spp. and *Cryptosporidium* spp. The strategy involves three basic steps: (i) concentration and filtration of the water sample to recover small numbers of *Toxoplasma* oocysts, (ii) elution and purification on a density gradient, and (iii) detection by PCR amplification and bioassay (mouse inoculation) to determine the presence and the infectivity of recovered oocysts. This strategy was applied to 139 water samples over a 20-month period.

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MATERIALS AND METHODS

Oocyst stock preparation. Oocysts were isolated from the feces of a cat experimentally infected with 3,000 cysts of a chronic type II strain (RMS/99/

TABLE 1. Water samples[a] and results of PCR and bioassay

Sample type	Vol. (liters)	No. of oocysts added	No. of oocysts per liter	No. of samples	No. of positive samples/total no. tested (% positive)	
					PCR	Bioassay
DW	100	1×10^3	10	10	10/10 (100)	6/10 (60)
DW	100	1×10^2	1	10	6/10 (60)	3/10 (30)
PDW	100	1×10^3	10	10	6/10 (60)	5/10 (50)
PDW	100	1×10^2	1	10	5/10 (50)	2/10 (20)
RSW	40	4×10^4	1,000	10	5/10 (50)	3/10 (30)
RSW	40	4×10^3	100	10	2/10 (20)	0/10 (0)
RSW	40	4×10^2	10	10	0/10 (0)	0/10 (0)

[a] The samples were *T. gondii* oocyst seeded, GES capsule filtered and eluted.

GUI). Nonsporulated *T. gondii* oocysts were purified and induced to sporulate as described by Dubey and Frenkel (10). Briefly, in step 1, the feces were emulsified in water and centrifuged; the supernatant was discarded, and sediment 1 was recovered. Step 2 consisted of mixing sediment 1 with 10 volumes of sucrose solution (specific gravity, 1.15) and centrifuging (1,250 × *g*). After step 2, oocysts were found in supernatant 2. In step 3, approximately 5 ml of supernatant 2 was mixed with 45 ml of water and then centrifuged (1,250 × *g*); the oocysts were now in sediment 3. Five milliliters of 2% aqueous H_2SO_4 was added to sediment 3, and the tube was aerated at room temperature for 7 days. Sporulated oocysts were stored at 4°C until used in seeding experiments.

Seeding. Samples of deionized water (DW), public drinking water (PDW), and raw surface water (RSW) (100 liters each) were seeded with known numbers of oocysts. The oocyst stock suspension was counted with a hemocytometer, and the mean value obtained for 10 different samples was analyzed. The stock suspension was adjusted to 10^5 oocysts/ml. DW and PDW (100 liters) were seeded with 10^2 or 10^3 oocysts, and RSW (40 liters; maximal filtration capacity) was seeded with 4×10^4, 4×10^3, or 4×10^2 oocysts.

Environmental samples. The sampling sites were selected by local public health officials in the four departments of the Champagne-Ardenne region. The survey was carried out over a 20-month period (June 2001 to January 2003). A total of 139 samples were analyzed, consisting of RSW collected near water plant intakes ($n = 45$), underground water (UW) ($n = 50$), and PDW ($n = 44$). Samples from different geographical locations exhibited a range of turbidities measured by the nephelometric procedure; 100 liters was collected in 10-liter polypropylene barrels at each source and transported to the laboratory for immediate processing. The filtered volumes were 100 liters of PDW, 25 to 100 liters of UW, and 7 to 45 liters of RSW. Some samples also contained *Giardia* spp. and *Cryptosporidium* spp., which were detected by indirect immunofluorescence after immunomagnetic separation (IMS), using method 1623 of the U.S. Environmental Protection Agency (USEPA) (15).

Oocyst detection. (i) Filtration. Each sample was concentrated using Envirochek capsules (Pall Gelman Laboratory) as specified by the manufacturer. We tested both the original Gelman Envirochek Standard (GES) (a polyethersulfonate membrane with a 1-μm absolute pore size, designed for 10 to 100-liter volumes) and Envirochek HV (GEHV) (a new 1-μm track-etched membrane for water volumes of 500 liters or more). All the samples were concentrated using GES capsules, and some (seeded DW) were also concentrated with GEHV capsules. Particulate matter was eluted from the capsule filter with elution buffer containing detergents (Tween 80 and Laureht-12; Pall Gelman Laboratory). Eluates were centrifuged (30 min at 1,250 × *g* and 4°C); distilled water was added to the pellet-eluting solution (adjusted to 10 ml), and the solution was subjected to IMS for detection of *Giardia* spp. and *Cryptosporidium* spp. After this IMS step, the eluate was centrifuged (10 min at 1,250 × *g* and 4°C), supernatant 1 was discarded and pellet 1 was suspended in sucrose suspension (density, 1.15; ratio: 1:3). After centrifugation (10 min at 1,250 × *g* and 4°C), 2 ml of supernatant 2 (which would contain oocysts) was collected and mixed with 8 ml of DW. After last centrifugation (10 min at 1,250 × *g* and 4°C), supernatant 3 was discarded and sediment 3 was used for *T. gondii* detection.

(ii) Detection. The sediment obtained after sucrose separation was separated into two parts. One was used for PCR, and the other was used for bioassay (mouse inoculation) after sporulation.

(a) PCR detection. The sediment was resuspended in 1 ml of Tris-EDTA buffer and centrifuged (3 min at 1,250 × *g*). Three cycles of freezing (−80°C)-thawing were done, and the pellet was subjected first to proteinase K digestion (1 h at 60°C) and then to DNA extraction with QIAamp DNA minikits (Qiagen, Courtaboeuf, France) as specified by the manufacturer. DNA was then subjected to real-time PCR for detection of the *T. gondii* B1 gene with the following primers described by Lin et al. (25) Toxo-F (5 μM; 5′-TCC CCT CTG CTG GCG AAA AGT-3′) and Toxo-R (5 μM; 5′-AGC GTT, CGT GGT CAA CTA TCG ATT G-3). The target DNA for real-time PCR amplification was the published sequence of the 35-fold repetitive B1 gene of the RH strain (6). Template DNA (5 μl) was added to a reaction mixture (final volume, 25 μl) containing 0.75 U of *Taq* DNA polymerase (Platinum; Bio-Rad), 20 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 200 μM each dGTP, dATP, and dCTP, 400 μM dUTP, 0.5 U of UDG, 12.5 pmol of each primer, and 5 pmol of TaqMan fluorescent probe (2 μM; 5′FAM-TCT GTG CAA CTT TGG TGT ATT CGC AG-3′TAMRA). TaqMan PCR runs were performed in triplicate with an ICycler device (Bio-Rad). After initial activation of *Taq* polymerase at 95°C for 5 min, 45 PCR cycles were run at 95°C for 15 s and 60°C for 1 min. As the standard curve constructed with 10-fold serial-dilutions of purified *T. gondii* tachyzoites, this PCR was considered an end-point assay and so not appropriate for oocyst quantification. The PCR amplification was considered positive when DNA was detected in the three wells. To detect PCR inhibitors, DNA from a mimetic plasmid insert (corresponding to the target on the B1 gene deleted of nucleotides) was added in a second run in all DNA environmental samples, amplified with others primers, and revealed with SYBRgreen. When inhibitors were present (plasmid insert unamplified), 8 μg of bovine serum albumin (BSA; Boehringer, Mannheim, Germany) was added to a new reaction mixture before amplification (PCR/BSA).

(b) Bioassay. We used a previously described procedure to induce the sporulation and infectivity of any *T. gondii* oocysts in the sediment (12). After addition of 5 ml of 2% H_2SO_4, the sediment was aerated at room temperature for 7 days. The suspension was neutralized by adding 3.3% sodium hydroxide and then centrifuged (10 min at 1,250 × *g* and 4°C). The supernatant was discarded, and the sediment was resuspended in 1.5 ml of normal saline containing antibiotics. Female Swiss-Webster mice (three mice per sample) were inoculated by gavage with well-mixed resuspended sample sediment. All mice were first confirmed to be seronegative for *T. gondii*. Blood was collected 6 weeks after infestation for serological analysis by high-sensitivity direct agglutination as previously described (31). Infection was demonstrated by seroconversion (specific immunoglobulin G antibody detection) and brain examination of sacrificed mice (2 months after inoculation).

RESULTS

The results of PCR and bioassay detection of oocysts seeded in DW, PDW, and RSW are summarized in Table 1. PCR success rates in DW were 60% for the detection of 1 oocyst/liter and 100% for 10 oocysts/liter. The detection rate was 60% for 10 oocysts/liter in PDW and 50% for 1,000 oocysts/liter in RSW. The sensitivity of the PCR assay ranged from less than 10 to more than 1,000 oocysts/liter, depending on the source of the seeded sample. PCR was more sensitive than mouse inoculation. The detection rate at the bioassay detection limit in DW (1 oocyst/liter; Table 1) with the GES capsule was 30%. GES and GEHV capsules were evaluated for the recovery of *T. gondii* oocysts (Table 2). A 100-liter volume of DW seeded with 10^3 oocysts was filtered at 1.5 liters/min using the two

TABLE 2. Comparison of the ability of GES and GEHV capsules to filter 100 liters of deionized water seeded with 10^3 *T. gondii* oocysts

Capsule	No. of oocysts added	PCR detection	No. of positive mice in bioassay/total no. inoculated
GES	10^3	+	2/3
	10^3	+	2/3
	10^3	+	0/3
	10^3	+	2/3
	10^3	+	0/3
GEHV	10^3	+	3/3
	10^3	−	1/3
	10^3	+	2/3
	10^3	+	0/3
	10^3	+	0/3

capsules. Samples were concentrated as described above and tested by PCR and bioassay. PCR success rates were 100% for the detection of 10 oocysts/liter in DW with the GES capsule, and 80% with the GEHV capsule. The positivity rate was 60% (three of five) with both capsules in the bioassay; two of five GES-filtered samples were totally negative in the bioassay, while only two of three mice were positive with the other three samples.

A total of 139 water samples from 80 sites were tested by PCR and mouse inoculation. The turbidity of RSW samples ranged from 0.30 to 30.1 nephelometric turbidity units, while that of PDW samples ranged from 0.03 to 1.9 units. Among the 86 samples not containing PCR inhibitors, 9 were positive for *Toxoplasma* DNA and 77 were negative (Table 3). The other 53 samples contained PCR inhibitors. PCR-BSA overcame this inhibition in 39 cases (73.5%), leading to the detection of *Toxoplasma* DNA in one further positive sample. Inhibitors were more frequent in RSW (29 of 45; 64.4%) than in UW (18 of 50; 36%) or PDW (6 of 44; 13.6%). After PCR-BSA, only RSW (10 of 45; 22.2%) and UW (4 of 50; 8%) samples remained uninterpretable (principally samples with high turbidity).

Finally, among the 125 interpretable samples, we detected *Toxoplasma* DNA in 10 cases (8%); 14 samples (10%; 10 RSW and 4 UW) were not amplified because of PCR inhibitors. None of the samples were positive by bioassay.

DISCUSSION

T. gondii oocysts can persist for long periods in the environment. Experimental ingestion of a single oocyst can infect mice and pigs (12) while no data have been reported for human toxoplasmosis. *T. gondii* oocysts also exhibit remarkable resistance to various inactivation procedures based on chemical reagents (8, 9) and disinfection processes used by water utilities (42). The different methods for *T. gondii* oocyst recovery were recently reviewed (14). Standard methods currently available for detection of *Cryptosporidium spp*. and *Giardia spp*. in water include concentration-filtration of large volumes, elution and clarification on density gradients or by IMS, and immunofluorescence assay (IFA) using monoclonal antibodies (32). Here, we describe a rapid method for *Toxoplasma* detection in environmental water, including detection of *Cryptosporidium* spp. and *Giardia* spp. in the same sample.

The concentration-filtration step is critical and results in a marked loss of seeded oocysts (30). Recently, two chemical flocculation procedures and one centrifugation procedure were evaluated by Kourenti et al. for *T. gondii* oocyst concentration on DW, with recovery rates of more than 80% (23). While flocculation is simple and inexpensive, filtration is more robust for processing turbid water. Moreover, samples concentrated by filtration are less likely to contain PCR inhibitors, which appeared to be eliminated by using GES filters (28). For *T. gondii*, since oocyst numbers in random environmental samples are probably small, we used GES capsules to filter 7 to 45 liters of RSW and 100 liters of PDW. Since GEHV capsules permit the filtration of larger volumes (up to 1,000 liters), we also tested them with *T. gondii*-seeded samples. No major difference was found between the two capsules, and GEHV could thus be considered interesting for filtering larger volumes potentially containing *T. gondii* oocysts. The concentration-filtration process leads to the accumulation of debris in the filter, and oocyst detection requires clarification procedures to separate oocysts from debris (34). We used sucrose flotation which is commonly used for *T. gondii* oocyst purification (9, 12), since no monoclonal antibodies against the *T. gondii* oocyst outer wall are currently available (ruling out the use of IMS for concentration and IFA for quantitative detection).

Rapid and sensitive pathogen detection methods are essential for the public-water industry. PCR was described as more rapid, sensitive, and specific for *Cryptosporidium* species detection in environmental water samples (17, 19, 21); the sensitivity was found to be comparable for TaqMan PCR and conventional IFA (7). For DNA extraction, Sluter et al. (38) showed that three cycles of freezing-thawing were sufficient to expose *Cryptosporidium* oocyst DNA, resulting in higher sensitivity than proteinase K digestion or sonication. For *T. gondii* DNA extraction, we chose freezing-thawing for its speed, low cost, and simplicity. Nevertheless, PCR inhibitors may not be completely removed by flotation procedures and can compromise the sensitivity of molecular detection (5). Kreader (24) and Rochelle et al. (33) showed that BSA can be added to the PCR mix to avoid inhibition in samples containing humic acids (mixtures of complex polyphenolics produced during the decom-

TABLE 3. DNA amplification by PCR or PCR-BSA in 139 samples[a]

Sample[a]	Amplification method[b]	Total no. of samples	No. of samples showing:		
			T. gondii DNA absent	*T. gondii* DNA present	Inhibitor persistence[c]
RSW	PCR	16	13	3	
	PCR-BSA	29	19		10
UW	PCR	32	26	6	
	PCR-BSA	18	14		4
PDW	PCR	38	38		
	PCR-BSA	6	5	1	
Total		139	115	10	14

[a] There were 45 RSW samples, 50 UW samples, and 44 PDW samples.
[b] The PCR-BSA method was used when inhibitors hindered standard PCR.
[c] Persistence after BSA treatment; result uninterpretable.

position of organic matter) (41). In our study, the use of PCR-BSA reduced the incidence of inhibitors from 38 to 10% of samples. The sensitivity of the PCR assay was reduced by up to 100-fold in oocyst-seeded RSW compared with DW and PDW. This is consistent with results from Johnson et al. (21) for PCR-based *Cryptosporidium* oocyst detection in water.

Finally, among the 125 interpretable samples, we detected *Toxoplasma* DNA in 10 cases (8%). Three cases involved RSW, whose environmental matrices may be contaminated by soil washing after peaks in rainfall (4). This could also be the case for the UW samples (six positive samples), which were chosen by local public health officials because of frequent pathogen recovery (including *Giardia* spp. and *Cryptosporidium* spp.). The detection of *Toxoplasma* DNA in PDW was more surprising, since none of the samples were positive by bioassay. In a previous study (20), *T. gondii* identification after filtration was based on mouse inoculation. Mouse bioassay is still the reference method to detect viable oocysts, but 7 days is required for sporulation, before mouse inoculation, and an additional 4 weeks is required to obtain the immunological results (20). As previously reported by Kourenti et al. (23), our seeding data suggest that *Toxoplasma* oocysts remain infective for mice. However, our bioassay results with environmental water samples were disappointing and showed that the efficiency of the bioassay for 10^3 oocyst-seeded, & filtered, and eluted water samples was poor. Dubey et al. (12) and Isaac-Renton et al. (20), using the same method, failed to detect *Toxoplasma* oocysts in PDW. Previously, it was reported that the different isolation steps and vigorous shaking necessary for consistent oocyst elution may cause a loss of infectivity (35, 39). All these results show that while the mouse bioassay is still the reference test for viable oocysts, its efficiency is poor when it is applied to filtered environmental water samples. This could explain why none of our *T. gondii* PCR-positive samples were positive by bioassay. Another cause could be DNA contamination or detection of DNA from noninfective oocysts present in the environment. To avoid contamination, DNA extraction, PCR mix preparation, and PCR amplification were done in separated rooms. TaqMan PCR minimizes the possibility of cross-contamination because it involves a closed-vessel system and because of the presence of UDG in the mix (7). To distinguish between DNA from living and from dead oocysts, reverse transcription-PCR could be used as an important indirect technique for oocyst viability determination while it selectively detects viable organisms.

In conclusion, we describe here a strategy for the detection of multiple waterborne parasites, including *Toxoplasma* oocysts, in environmental water samples. PCR amplification was able to detect *Toxoplasma* DNA, even when the mouse bioassay was negative. These results suffer from some limitations, mainly owing to the flotation step. The development of monoclonal antibodies against the *Toxoplasma* oocyst wall would permit the use of IMS and also microscopic quantification by IFA. Moreover, efforts are under way in our laboratory to develop a quantitative reverse transcription-PCR method to differentiate between viable and nonviable oocysts detected in environmental samples since the mouse bioassay takes too long for public health sentinel purposes.

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